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Oxidation of Morphine to 2,2′-Bimorphine by *Cylindrocarpon didymum*

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The oxidation of morphine by whole-cell suspensions and cell extracts of *Cylindrocarpon didymum* gave rise to the formation of 2,2′-bimorphine. The identity of 2,2′-bimorphine was confirmed by mass spectrometry and ^{1}H nuclear magnetic resonance spectroscopy. *C. didymum* also displayed activity with the morphine analogs hydromorphone, 6-acetylmorphine, and dihydromorphine, but not codeine or diamorphine, suggesting that a phenolic group at C-3 is essential for activity.

The morphine alkaloids are the major alkaloid components of opium, the dried latex material from cut seed capsules of the opium poppy, *Papaver somniferum*. Of all the alkaloids, the morphine alkaloid group has been studied in most detail, mainly due to the significant therapeutic properties these compounds possess. The morphine alkaloids are narcotic analgesics and are widely used by clinicians for the control of chronic pain. The use of microbial enzymes to provide biological routes for the synthesis of semisynthetic drugs that are difficult to synthesize chemically and as a means of producing new morphine alkaloid derivatives has been the subject of a significant amount of research; this topic has recently been reviewed (3). In recent years, there has been an increasing demand for new morphine alkaloid intermediates for the synthesis of novel semisynthetic drugs, and as part of a study to produce such compounds, we have been exploring fungal transformations of morphine. In this paper, we describe the conversion of morphine to pseudomorphine (2,2′-bimorphine) by *Cylindrocarpon didymum* 311186.

Biotransformation of morphine. *C. didymum* 311186 was obtained from the International Mycological Institute (Egham, Surrey, United Kingdom). Mycelia were grown in media at pH 7.0 containing (grams per liter) yeast extract (10.0), KH_2PO_4 (10.0), $(NH_4)_2SO_4$ (5.0), and $MgSO_4$ (0.5). Trace elements were as described by Rosenberger and Elsden (9). Cultures were incubated at 30°C for 48 h with rotary shaking at 180 rpm. Washed mycelia (typically 0.5 g [wet weight]) were resuspended in 40 ml of medium containing 10 mM morphine (Macfarlan Smith Ltd., Edinburgh, United Kingdom) in 250-ml Erlenmeyer flasks. Samples (0.2 ml) were removed at regular intervals and diluted fivefold in 50 mM phosphoric acid (pH 3.5), to dissolve any insoluble metabolites. Mycelia were removed by centrifugation at 14,000 × *g* with an MSE Microcentaur microcentrifuge (Patterson Scientific Ltd., Dunstable, United Kingdom). The samples were analyzed by high-performance liquid chromatography (HPLC) with a Waters component system (Millipore Waters UK Ltd., Watford, United Kingdom). The HPLC system consisted of a 600E system controller connected to either a 484 absorbance detector or a model 994 programmable photodiode array detector set to 230 nm, 0 to 1 V full-scale detection. Injections of 50 μl were performed with a WISP 712 autoinjector and data processed with Millennium 2010 software. Separation of samples was achieved with a C_{18} Spherisorb column (4.6 by 250 mm, 5-μm particle size; Anachem Ltd., Luton, United Kingdom), protected by a guard column of the same packing material with an isocratic solvent system containing 40 mM phosphoric acid buffer (pH 2.5) and acetonitrile in a ratio of 92:8 plus 2 mM pentanesulfonic acid, delivered at a flow rate of 1 ml/min. Analysis of the whole-cell incubation mixture by HPLC showed that morphine was completely removed from the medium after a period of 70 h. No other soluble metabolites were identified by HPLC; however, a white precipitate was found to accumulate in the incubation mixture. Microscopic analysis showed that the precipitate had formed regular cubic crystals. The crystalline material was found to dissolve under mildly acidic conditions, and HPLC analysis after such treatment revealed the stoichiometric conversion of morphine to an unknown compound (Fig. 1) that had a retention time that coincided with that of authentic 2,2′-bimorphine, kindly provided by M. McPherson (Macfarlan Smith Ltd.). The compound was analyzed by thin-layer chromatography (TLC) with polyester-backed plates precoated with Polygram Sil G/UV_{254} (Machery-Nagel, Duren, Germany) and developed in ammonia-*n*-butanol (20/80 [vol/vol]). TLC analysis revealed the appearance of two compounds that were detectable under UV light at 254 nm and with Ludy Tenger reagent (7). Compound 1 had an R_f value of 0.42 corresponding to that of authentic morphine, while compound 2 had an R_f value of 0.25 which coincided with that of authentic 2,2′-bimorphine. 2,2′-Bimorphine shows greatly enhanced fluorescence characteristics, compared to those of morphine, due to extended conjugation (6). Compound 2 fluoresced with a characteristic blue color when the TLC plate was illuminated at 366 nm. Fluorescent excitation and emission spectra of compound 2 dissolved in 50 mM potassium phosphate buffer (pH 7.4) in 1-cm-path-length cuvettes were recorded with a Perkin-Elmer LS 50 B luminescence spectrometer (Perkin-Elmer Ltd., Beaconsfield, United Kingdom). Two principal excitation maxima were found at 280 and 320 nm, with a single emission maximum at 430 nm, typical of authentic 2,2′-bimorphine.

Identification of 2,2′-bimorphine. ^{1}H nuclear magnetic resonance spectroscopy of the product was performed at 400 MHz with a Bruker AM-400 spectrometer with tetramethylsilane as an internal standard and D-6 dimethyl sulfoxide as the solvent. The ^{1}H nuclear magnetic resonance spectrum gave the following signals, which were indistinguishable from those of an authentic sample of 2,2′-bimorphine (5) (for the proton assignments, see Fig. 2, which gives the 2,2′-bimorphine numbering system): δ H 6.31 (2H, s, 1-H and 1′-H); 5.58 (2H, dd, J = 9.6 and 2.5, 7-H and 7′-H); 5.26 (2H, d, J = 9.6, 8-H and 8′-H); 4.70 (2H, d, J = 5.7, 5-H and 5′-H); 4.10 (2H, dd, J = 5.7

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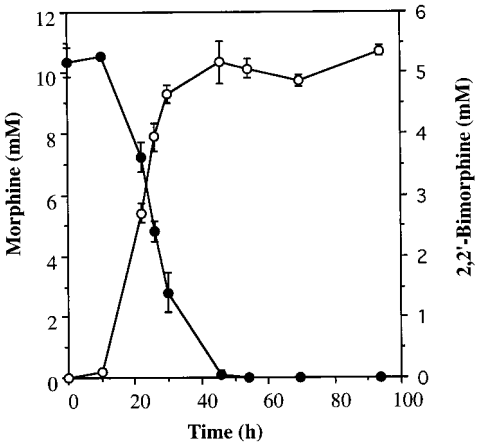

FIG. 1. Accumulation of 2,2′-bimorphine in whole-cell incubations of *C. didymum*. Whole-cell incubations contained 40 ml of minimal medium, 10 mM morphine, and 0.5 g (wet weight) of mycelia in 250-ml Erlenmeyer flasks. Morphine (●) and 2,2′-bimorphine (○) concentrations were determined by HPLC. The data are means of three replicate incubations.

and 2.5, 6-H and 6′-H); 3.29 (2H, dd, J = 6.2 and 2.6, 9-H and 9′-H); 2.91 (2H, d, J = 18.6, 10β-H and 10β′-H); 2.57 (2H, d, J = 2.6, 14-H and 14′-H); 2.50 (2H, dd, J = 12.5 and 3.5, 16β-H and 16β′-H); 2.32 (6H, s, NMe and NMe′); 2.28 (2H, d, J = 12.5, α16-H and α16′-H); 2.23 (2H, dd, J = 18.6 and 6.2, α10-H and α10′-H); 1.99 (2H, dd, J = 11.4 and 3.5, α15-H and α15′-H); 1.68 (2H, d, J = 11.4, β15-H and β15′-H).

The ^{1}H spectrum agreed with that expected for a symmetrical dimer, and only one aromatic proton signal was observed, instead of the characteristic AB pair of the morphine spectra, suggesting a symmetrical substitution on the aromatic ring. Laser desorption time-of-flight mass spectrometry was performed with a Kompact Maldi III mass spectrometer, and the mass spectrum showed a molecular ion, m/z 569.4, for $C_{34}H_{36}N_2O_6$.

Transformations of morphine analogs by *C. didymum*. Whole-cell incubations of *C. didymum* were challenged with a range of morphine analogs including hydromorphone, 6-acetylmorphine, dihydromorphine, codeine, and diamorphine (see Fig. 2 for structures). The incubations contained in 250-ml Erlenmeyer flasks approximately 0.61 g (wet weight) of mycelia and morphine analogs at 5 or 10 mM in a total volume of 40 ml of

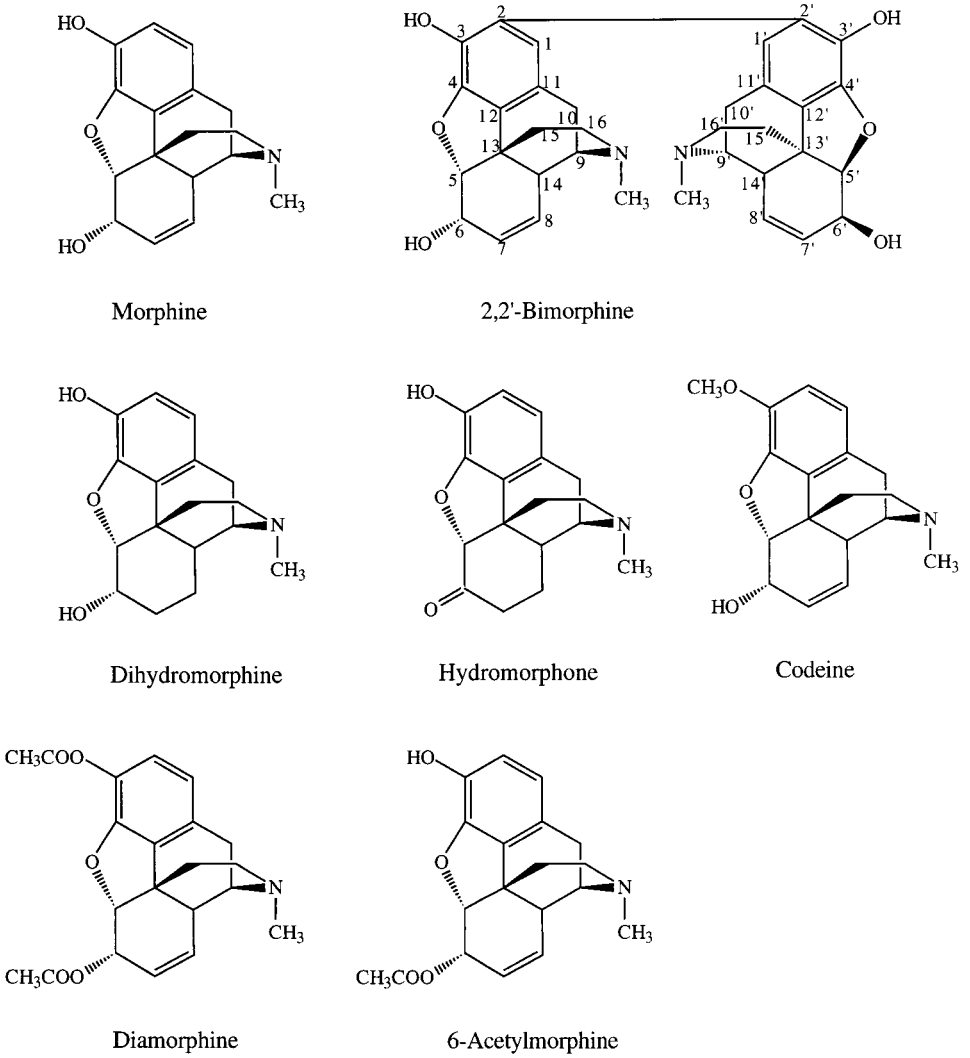

FIG. 2. Morphine analogs.

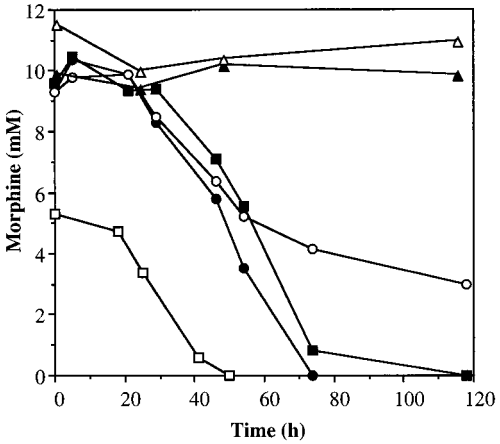

FIG. 3. Transformations of morphine analogs by *C. didymum*. Whole-cell incubations contained 40 ml of minimal medium, 10 mM substrate (5 mM dihydromorphine), and 0.61 g (wet weight) of mycelia in 250-ml Erlenmeyer flasks. Morphine (●), codeine (△), diamorphine (▲), hydromorphone (○), dihydromorphine (□), and 6-acetylmorphine (■) concentrations were determined by HPLC.

minimal medium. The flasks were incubated at 30°C with shaking, and samples were removed at intervals for HPLC analysis. Figure 3 shows that *C. didymum* was capable of activity with morphine, hydromorphone, 6-acetylmorphine, and dihydromorphine, and precipitates were observed to accumulate. Structural information on these products was not obtained. All of these compounds possess a free phenolic group at C-3 as a common structural feature which is likely to be an essential requirement for activity. This is consistent with the chemical oxidation of morphine to 2,2′-bimorphine, which requires the formation of a phenoxy radical intermediate (1).

Enzyme activity in cell extracts. The whole-cell transformation of morphine to 2,2′-bimorphine prompted investigation of subcellular enzyme activity. Cell extract was prepared by the method of Rahim and Sih (8) with the following modifications. Frozen mycelia containing 10 to 14 g (wet weight) of biomass were placed in an ice-cold mortar with an equal weight of acid-washed white quartz sand (50/70 mesh; Sigma Chemical Company, Poole, United Kingdom) and an equal volume of ice-cold potassium phosphate buffer (pH 7.4). The mixture was ground with a pestle for approximately 20 min until it formed a thin paste. The paste was diluted with an equivalent volume of ice-cold buffer, and the sand and cell debris were removed by centrifugation at 20,000 × *g* for 15 min at 4°C in a Sorvall RC5C centrifuge fitted with an SS34 rotor. Protein was measured by the method of Bradford (2) with the Pierce protein assay reagent according to the manufacturer's protocol. Typically, protein recoveries of approximately 7 mg of protein/g (wet weight) of cells were obtained. The fluorescent nature of 2,2′-bimorphine enabled the development of a convenient and sensitive enzyme assay. In reaction mixtures which contained potassium phosphate buffer (pH 7.4), morphine (5 mM), and cell extract, activity could be measured spectrofluorimetrically by measuring fluorescence of 2,2′-bimorphine at 440 nm when excited at 330 nm. Cell extract from mycelia harvested after 80 h of incubation with morphine had a specific activity of 0.36 U/mg of protein. One unit of activity was defined as the amount of enzyme required to produce 1 μmol of 2,2′-bimorphine from 2 μmol of morphine per min. No activity was observed in control reaction mixtures where the cell extract was replaced with boiled cell extract. Activity was inhibited completely when 0.1 mM azide was added to the reaction mixtures. Interestingly, no activity was observed in cell extract from mycelia that had not been incubated with morphine, which suggests that the activity is inducible. The development of a rapid and sensitive assay should facilitate the purification and characterization of the 2,2′-bimorphine-producing enzyme. 2,2′-Bimorphine has been shown to be a spontaneous reaction product of morphine in aqueous solutions, though the reaction was extremely slow (4). Furthermore, morphine can be oxidized to 2,2′-bimorphine with alkaline ferricyanide, a reaction which is known to proceed via a mesomeric aryloxy free radical, leading to the formation of the dimer (1). However, to the best of our knowledge, this is the first report of the microbial oxidation of morphine to 2,2′-bimorphine.

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